Chryseobacterium formosense sp. nov., isolated from the rhizosphere of *Lactuca sativa* L. (garden lettuce)

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A yellow-pigmented bacterial strain (CC-H3-2^T), isolated from the rhizosphere of *Lactuca sativa* L. (garden lettuce) in Taiwan, was investigated using a polyphasic taxonomic approach. The cells were Gram-negative, rod-shaped and non-spore-forming. Phylogenetic analyses using the 16S rRNA gene sequence of the isolate indicated that the organism belongs to the genus *Chryseobacterium*, with the highest sequence similarity to the type strains of *Chryseobacterium indoltheticum* (97.7%), *Chryseobacterium scophthalmum* (97.5%), *Chryseobacterium joostei* (97.2%) and *Chryseobacterium defluvii* (97.2%). The major whole-cell fatty acids were iso-C_{15:0} (52.2%) and iso-C_{17:0} 3-OH. DNA–DNA hybridization experiments revealed levels of only 27.4% to *C. scophthalmum*, 27.1% to *C. indoltheticum*, 14.1% to *C. joostei* and 7.8% to *C. defluvii*. DNA–DNA relatedness and biochemical and chemotaxonomic properties demonstrate that strain CC-H3-2^T represents a novel species, for which the name *Chryseobacterium formosense* sp. nov. is proposed. The type strain is CC-H3-2^T (= CCUG 49271^T = CIP 108367^T).

At the time of writing, the genus *Chryseobacterium* comprises ten species: *Chryseobacterium balustinum*, *Chryseobacterium gleum*, *Chryseobacterium indologenes*, *Chryseobacterium indoltheticum*, *Chryseobacterium menin-gosepticum*, *Chryseobacterium miricola*, ‘*Chryseobacterium proteolyticum*’ (name not validly published), *Chryseobacterium scophthalmum*, *Chryseobacterium joostei* and *Chryseobacterium defluvii*. These species and the taxonomy of the genus have been extensively described in recently published papers (Hugo *et al.*, 2003; Li *et al.*, 2003; Kämpfer *et al.*, 2003).

Although several strains of *Chryseobacterium* species have recently been well characterized with regard to their ability to produce heat-stable metalloproteases and protein-deamidating enzymes (Venter, 1987; Yamaguchi & Yokoe, 2000), their functional role in agricultural soil or plant growth promotion remains largely unclear. While screening for proteolytic activity on skimmed milk agar, five different rhizobacteria from *Lactuca sativa* L. (garden lettuce) were selected that showed strong proteolytic

activities. An additional isolate from the rhizosphere of *Lactuca sativa* L. from Kuohsing, Taiwan, revealed a yellow pigmentation on nutrient agar. This strain (CC-H3-2^T) was maintained and subcultivated on brain heart infusion agar at 30 °C for 48 h and subsequently investigated via 16S rRNA gene sequence analysis. We determined the fatty acid methyl ester composition of whole cell hydrolysates, further phenotypic characteristics [carbon source utilization, API ZYM enzyme profiles (bioMérieux), API 20E tests (bioMérieux)] and DNA–DNA relatedness to those species most closely related on the basis of 16S rRNA gene sequence similarity.

Cultural and morphological characteristics were observed on nutrient agar and brain heart infusion agar. Flexirubin-like pigments were observed by flooding the plates with 20% (w/v) potassium hydroxide (Fautz & Reichenbach, 1980). Gram reaction was tested by the modified method of Cowan (1974) and motility was tested microscopically from cells grown for 3 days in motility broth at 30 °C (Cowan, 1974). Fluorescence was tested after plating on King’s B medium after 48 h (Cowan, 1974).

Strain CC-H3-2^T was Gram-negative and formed visible (about 2 mm) yellowish colonies after 48 h growth at 30 °C. No growth was observed above 37 °C, whereas very small colonies were visible after prolonged incubation at

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15 °C. Within 48 h at 20 °C, clear, visible colonies appeared. The colonies were translucent and shiny with entire edges, but on prolonged incubation the colonies were not visible as single entities, probably owing to the profuse production of extracellular substances. A bright yellow, non-diffusible, non-fluorescent flexirubin pigment was produced on nutrient agar. Oxidase activity was tested using oxidase reagent (bioMérieux) according to the manufacturer's instructions. Cells of strain CC-H3-2^T were oxidase-positive, non-motile, non-spore-forming rods (1 µm wide, 2 µm long). Strain CC-H3-2^T was able to grow well on nutrient agar, trypticase soy agar and brain heart infusion agar but was unable to grow on MacConkey's agar.

Physiological characterization and additional biochemical tests were performed to assess the pattern of carbon source utilization and hydrolysis of 19 substrates using the API ZYM system and API 20E according to the methods outlined by the manufacturer (bioMérieux).

Analysis of the cellular fatty acid composition (method as described by Kämpfer *et al.*, 2003) showed that iso-C_{15:0} was the most abundant fatty acid (52.2%) followed by iso-C_{17:0} 3-OH (10.9%) and summed feature 4 (iso-C_{15:0} 2-OH/C_{16:1}ω7t, 6.5%). The fatty acid pattern for strain CC-H3-2^T is shown in Table 1 in comparison to all other

Chryseobacterium species. The profile for strain CC-H3-2^T was typical of *Chryseobacterium* species, and most similar to that of *C. defluvii*.

The 16S rRNA gene sequence was amplified by PCR using universal primers 1F (5'-GAGTTTGATCATGGCTCAG-3') and 7R (5'-TGACGGGCGGTGTGTACAA-3') (Edwards *et al.*, 1989). Sequence analysis was performed using a DNA sequencer (ABI PRISM 310; Applied Biosystems), sequence assembly was performed using the Wisconsin package version 9.1 (GCG, Inc.) with the Fragment Assembly System program supplied by the National Health Research Institute of Taiwan and a phylogenetic tree was constructed using the neighbour-joining method from the distance matrices. Maximum-parsimony and neighbour-joining methods (Fig. 1) were also applied for tree construction.

The nearly full-length (1416 nt) 16S rRNA gene sequence of strain CC-H3-2^T was aligned with sequences deposited in GenBank by using CLUSTAL W software available from the European Bioinformatics Institute. Searches indicated that strain CC-H3-2^T was phylogenetically closely related to species of the genus *Chryseobacterium*. The most closely related species was *C. indoltheticum* (97.5%) followed by *C. scopthalmum* (97.2%), *C. defluvii* (97.2%), *C. joostei*

Table 1. Long-chain fatty acid composition of *Chryseobacterium* species and related bacteria

Taxa: 1, *C. formosense* sp. nov. (n=1); 2, *C. defluvii* (n=1); 3, *C. joostei* (n=11); 4, *C. gleum* (n=5); 5, *C. indologenes* (n=45); 6, *C. balustinum* (n=1); 7, *C. indoltheticum* (n=1); 8, *C. scopthalmum* (n=2); 9, *C. meningosepticum* (n=1); 10, *C. miricola* (n=1); 11, *Bergeyella zoohelcum* (n=1); 12, *Empedobacter brevis* (n=6). All strains identified as *C. gleum*, *C. indologenes* and *Empedobacter brevis* in an earlier study (Hugo *et al.*, 2003) were included. Fatty acids amounting to less than 1% of the total fatty acids in all strains were not included. Values are given as means±SD. tr, Trace (less than 1.0%); ND, not detected. Data from Kämpfer *et al.* (2003), Hugo *et al.* (2003) and Li *et al.* (2003).

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12
iso-C _{13:0}	3.6	2.8	tr	tr	tr	tr	ND	tr	1.4	tr	1.5	tr
Unknown 13:566*	tr	tr	1.1±0.2	1.2±0.4	2.1±0.7	1.6	1.7	2.9±0.2	1.5	tr	1.8	1.9±1.4
iso-C _{15:0}	52.2	58.5	34.6±2.0	35.4±2.9	34.3±4.9	32.3	29.4	35.0±0.7	41.4	33.0	47.8	23.6±2.2
iso-C _{15:0} 3-OH	1.8	2.6	2.9±0.3	2.5±0.1	2.6±0.2	2.7	2.3	2.7±0.1	3.5	3	3.95	4.8±0.7
anteiso-C _{15:0}	2.1	3.2	tr	tr	tr	tr	5.9	tr	2.3	tr	ND	tr
C _{16:0}	1.5	1.3	tr	1.3±0.4	tr	1.6	1.0	1.2±0.2	tr	2	tr	3.8±0.4
C _{16:0} 3-OH	tr	tr	1.2±0.2	1.1±0.1	1.0±0.2	1.4	tr	1.0±0.1	2.2	8r	ND	3.6±0.7
iso-C _{16:0} 3-OH	1.1	tr	ND	ND	ND	tr	1.3	ND	tr	tr	ND	tr
Unknown 16:580*	1.0	ND	1.6±0.1	1.7±0.1	1.7±0.2	1.3	1.3	1.5±0.1	1.7	tr	1.4	1.4±0.1
C _{17:0} 2-OH	ND	ND	ND	ND	ND	tr	3.0	tr	tr	ND	ND	tr
iso-C _{17:0}	2.3	2.0	tr	1.6±0.6	tr	1.0	tr	tr	tr	1	ND	tr
iso-C _{17:0} 3-OH	10.9	14.1	20.1±1.2	21.8±0.3	19.2±1.8	16.8	14.0	16.3±0.1	16.3	40	13.5	17.1±2.1
iso-C _{17:1} ω9c	4.3	4.8	22.9±1.9	20.2±3.9	24.2±3.1	27.1	25.6	24.8±0.4	7.0	tr	17.5	3.9±0.5
C _{18:1} ω5c	ND	ND	tr	tr	tr	tr	tr	tr	tr	ND	1.5	ND
Summed feature 4†	6.5	8.4	12.1±1.3	11.8±0.8	11.1±1.3	9.2	11.2	11.5±0.3	18.1	12	9.0	19.4±1.6
Summed feature 5†	ND	ND	tr	tr	tr	tr	ND	tr	tr	tr	1.5	6.7±0.8

*Unknown fatty acid; numbers indicate equivalent chain length.

†Fatty acids that could not be separated by GC using the Microbial Identification System (Microbial ID) software were considered summed features. Summed feature 4 contains iso-C_{15:0} 2-OH and/or C_{16:1}ω7t; summed feature 5 contains iso-C_{17:1} I and/or anteiso-C_{17:1} B.

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from the EMBL database (accession numbers are given in parentheses) constructed after multiple alignment of data by CLUSTAL X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model) were determined and clustering with the neighbour-joining method was performed by using the software package MEGA version 2.1 (Kumar *et al.*, 2001). Bootstrap values based on 1000 replications are listed as percentages at branch points. Bar, 0.02 substitutions per nucleotide position.

(97.2 %) and *C. indologenes* (97.1 %); sequence similarities to all other *Chryseobacterium* species were below 96.8 %.

DNA–DNA hybridization experiments were performed with strain CC-H3-2^T and the type strains of the four most closely related *Chryseobacterium* species using the method described by Ziemke *et al.* (1998) except that, for nick translation, 2 µg DNA was labelled with 3 h of incubation at 15 °C. Strain CC-H3-2^T showed relatively low levels of DNA–DNA relatedness to the type strains of *C. indoltheticum* CIP 103168^T (27.1 %, reciprocal 14.1 %),

C. scophthalmum CIP 104199^T (27.4 %, 15.7 %), *C. joostei* CIP 105533^T (14.1 %, 7.3 %) and *C. defluvii* B2^T (7.8 %). Pooled standard deviations of all hybridization experiments were between 8.0 and 11.6 %.

Strain CC-H3-2^T utilized only a few carbon sources but was able to hydrolyse 11 of 19 compounds in the API ZYM system. Biochemical/physiological characteristics of the strain are given in Table 2 and under the species description. A striking difference was observed in enzyme activities, especially for α -glucosidase and β -glucosidase.

Table 2. Comparison of characteristics of strain CC-H3-2^T with other recognized *Chryseobacterium* species

Species/strains: 1, *C. formosense* sp. nov. CC-H3-2^T; 2, *C. defluvii* (*n*=1); 3, '*C. proteolyticum*' (*n*=2); 4, *C. gleum* (*n*=12); 5, *C. indologenes* (*n*=13); 6, *C. balustinum* (*n*=1); 7, *C. indoltheticum* (*n*=1); 8, *C. meningosepticum* (*n*=49); 9, *C. scophthalmum* (*n*=7); 10, *C. joostei* (*n*=11); 11, *C. miricola* (*n*=1). Data for reference species were taken from Kämpfer *et al.* (2003), Li *et al.* (2003) and Hugo *et al.* (2003). +, All strains positive; (+), weakly positive; –, all strains negative; v, variable; NA, not available; two figures separated by a solidus (/) refer to the number of positive strains/number of strains tested.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Acid production from:*											
Glucose	(+)	+	+	+	+	+	+	+	–	+	+
Sucrose	–	–	+	–	–	–	–	–	–	–	–
Mannitol	–	–	+	–	4/13	–	–	31/49	–	+	+
Arabinose	–	–	+	10/12	–	–	–	1/49	–	–	–
Cellobiose	–	(+)	–	–	–	–	–	4/49	–	–	–
Fructose	NA	NA	NA	+	+	+	–	28/49	–	+	+
Lactose	NA	NA	–	–	–	–	–	27/49	–	–	+
Maltose	–	+	+	+	+	–	+	46/49	–	+	+
Trehalose	+	+	+	+	+	–	–	42/49	–	+	+
Xylose	(+)	–	NA	4/12	–	–	–	3/49	–	–	–
Casein digestion	NA	NA	+	+	NA	+	+	+	–	+	NA
Aesculin hydrolysis	+	+	+	+	+	–	+	47/49	–	+	+
Growth at 36–37 °C	–	+	+	+	+	–	+	+	–	–	+
Growth on MacConkey agar	–	–	–	+	v	+	+	+	–	+	(+)
Nitrate reduction	–	–	–	7/12	v	+	–	–	–	–	–
Urease activity	–	–	–	7/12	–	–	–	18/49	+	+	+
Indole production	+	+	+	+	+	+	+	24/49	–	+	+
Malonate utilization	–	–	–	NA	+	NA	NA	NA	NA	–	NA

*The methods for testing acid formation from different sugars are different. Readers should refer to the original references for direct comparisons.

C. defluvii and *C. scopthalmum* were positive for α -glucosidase but were negative for β -glucosidase activity whereas strain CC-H3-2^T was positive for β -glucosidase and negative for α -glucosidase activity.

On the basis of the results of this polyphasic study, it is evident that strain CC-H3-2^T represents a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium formosense* sp. nov. is proposed.

Description of *Chryseobacterium formosense* sp. nov.

Chryseobacterium formosense [for.mo.sen'se. N.L. neut. adj. *formosense* pertaining to Formosa (Taiwan)].

Cells are Gram-negative, non-motile, non-spore-forming rods (about 2 μ m in length and 1 μ m wide). Aerobic, oxidase-positive, with good growth after 48 h on trypticase soy agar, brain heart infusion agar and nutrient agar at 25–32 °C, but unable to grow on MacConkey's agar. Colonies on nutrient agar are smooth, yellowish, circular, translucent and shiny with entire edges; they become mucoid and cannot be identified as single entities after prolonged incubation. Yellow pigmentation is non-diffusible, non-fluorescent of flexirubin type. Unable to grow at 5 °C or above 37 °C. Major cellular fatty acids are iso-C_{15:0} and iso-C_{17:0} 3-OH. Positive for indole and acetoin production, gelatinase and cytochrome oxidase activity, but negative for β -galactosidase, arginine dihydrolase, lysine decarboxylase, citrate utilization, H₂S production, urease, tryptophan deaminase and oxidation of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. The following compounds are utilized as sole carbon sources: dextrin, D-arabitol, lactulose, acetic acid, D-sorbitol, sucrose, turanose, xylitol, citric acid, D-gluconic acid, D-glucosaminic acid, DL-lactic acid, quinic acid, succinamic acid, L-alaninamide, L-pyrogutamic acid (all weakly positive), cellobiose, gentiobiose, α -D-glucose, maltose, D-mannose, L-rhamnose, D-trehalose, monomethyl succinate, D-galacturonic acid, α -ketobutyric acid, α -ketovaleric acid, DL-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, inosine, uridine, thymidine, glucose 1-phosphate and glucose 6-phosphate. The following are not utilized as sole carbon sources: methyl β -D-glucoside, D-raffinose, propionic acid, cyclodextrin, glycogen, Tweens 40 and 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, *i*-erythritol, D-fructose, L-fucose, D-galactose, *m*-inositol, α -D-lactose, D-mannitol, D-melibiose, D-psicose, methylpyruvate, *cis*-aconitic acid, formic acid, D-galactonic acid lactone, D-glucuronic acid, α -, β - and γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketoglutaric acid, malonic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, glucuronamide, D-alanine, L-histidine, hydroxyproline, D-serine, DL-carnitine, γ -aminobutyric acid, urocanic acid, phenylethylamine,

putrescine, 2-aminoethanol, 2,3-butanediol, glycerol and DL- α -glycerol phosphate. Positive enzyme activities are seen for alkaline phosphatase, butyrate esterase, caprylate esterase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -glucosidase and N-acetyl- β -glucosaminidase, but negative for myristate lipase, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, α -mannosidase and α -fucosidase.

The type strain, CC-H3-2^T (= CCUG 49271^T = CIP 108367^T), was isolated from the rhizosphere of *Lactuca sativa* L. (garden lettuce).

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